Proteasome inhibitors induce apoptosis in growth hormone- and prolactin-secreting rat pituitary tumor cells

R Yu, S-G Ren and S Melmed

Cedars-Sinai Research Institute-UCLA School of Medicine, 8700 Beverly Blvd, Los Angeles, California 90048, USA
(Requests for offprints should be addressed to S Melmed; Email: melmed@csmc.edu)

Abstract

Proteasome inhibitors induce apoptosis in some malignant cells, and we show here that these inhibitors induce apoptosis in rat pituitary MMQ and GH3 tumor cells but not in normal pituitary cells. Three proteasome inhibitors, PSI, MG-132, and lactacystin, but not the calpain inhibitor, ALLM, dose- and time-dependently caused apoptosis in these cells, and 10 μ M PSI caused apoptosis in 70% of MMQ cells and in 25% of GH3 cells within 24 h. A lower PSI dose (10 nM) inhibited GH3 cell growth without causing significant apoptosis or affecting prolactin secretion. Primary rat pituitary cells were resistant to both PSI and MG-132 and did not undergo apoptosis. In MMQ cells, DNA synthesis was slowed ($\sim$ 30%) after 6 h

of 10 μ M PSI treatment and a partial cell cycle block at G2/M was evident after 8 h. Colorimetric caspase substrate assay and Western blotting of caspase substrates showed that caspases 2 and 3 are activated by PSI while caspases 6 and 8 remained inactive. A broad-range caspase inhibitor, caspase inhibitor III, prevented apoptosis induced by PSI. The results show that proteasome inhibitors induce apoptosis in rat pituitary tumor cells by specific caspase activation. This novel group of drugs may potentially be used in treatment of aggressive pituitary tumors, especially as their action appears relative for tumor cells.

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Introduction

Pituitary tumors are managed by surgery, radiation, and medical treatments (Shimon & Melmed 1998). Although tumors may be excised by surgery or ablated by radiation, these therapies are not effective for invasive tumors and they also have untoward side effects including damage to normal pituitary cells leading to hypopituitarism. With the development of dopaminergic and somatostatin analogs, hormone-secreting pituitary tumors are increasingly managed by medical treatment (Shimon & Melmed 1998). Dopaminergic agonists inhibit prolactin (PRL) secretion and shrink prolactinomas. Somatostatin analogs effectively inhibit growth hormone secretion but do not appreciably reduce tumor size. It is thus important to characterize agents that inhibit pituitary tumor growth or selectively ablate pituitary tumor cells. The anti-estrogens, tamoxifen and ICI 182 780, the potassium channel blocker, tetraethylammonium chloride, and leptin inhibit MMQ or GH3 tumor cell proliferation or cause tumor cell apoptosis (Lee *et al.* 1998, Vaur *et al.* 1998, Jin *et al.* 2000, Heaney *et al.* 2001).

Recently, a new group of antitumor agents, proteasome inhibitors, has been characterized (Drexler 1997, Lopes *et al.* 1997). Proteasomes are large, multimeric protease complexes that degrade polyubiquitinated proteins (Voges

et al. 1999, Pickart 2000). Several proteins, including tumor suppressors (e.g. p53, p21), cyclins, nuclear factor kappaB (NFkB), and mitosis-regulating proteins (e.g. cyclin B, securin), are degraded by the ubiquitin/proteasome mechanism (Hershko 1999, McDade *et al.* 1999, Voges *et al.* 1999, Zou *et al.* 1999, Pickart 2000, Yu *et al.* 2000b, Cusack *et al.* 2001, Russo *et al.* 2001). Proteasome inhibitors induce apoptosis in several tumor cell types including leukemia, prostate, pancreatic and lung cancers (Adams *et al.* 1999, McDade *et al.* 1999, Soligo *et al.* 2000, Sun *et al.* 2001). Caspases, mediators of apoptosis (Cryns & Yuan 1998, Thornberry & Lazebnik 1998), are often activated (Drexler 1997, Adams *et al.* 1999, Soligo *et al.* 2000, Sun *et al.* 2001), although the primary trigger of apoptosis is still unclear. Since the ubiquitin/proteasome pathway degrades several proteins, mechanisms for apoptosis may be cell-specific.

In this study, we examined the effects of proteasome inhibitors on rat pituitary MMQ and CH3 tumor cells. We found that proteasome inhibitors induced apoptosis, decreased DNA synthesis, and activated specific caspases in these cells. We also found that endocrine cells in primary rat pituitary culture were resistant to proteasome inhibitors. These results may indicate a potential application of proteasome inhibitors to selectively treat pituitary tumors while maintaining endogenous pituitary function.

Materials and Methods

Cells and reagents

GH3 cells were grown in monolayer in DMEM medium supplemented with 10% fetal bovine serum. MMQ cells were grown in suspension in RPMI 1640 medium supplemented with 15% horse serum and 2.5% fetal bovine serum. Primary rat pituitary cultures were established as described by Akita *et al.* (1995). The proteasome inhibitors PSI, MG-132, and lactacystin, the calpain inhibitor ALLM, the colorimetric caspase substrate set, and caspase inhibitor III were from Calbiochem (La Jolla, CA, USA). PSI was dissolved in methanol as a 10 mM stock; MG-132 (10 mM), lactacystin (5 mM), ALLM (10 mM), and caspase inhibitor III (10 mM) were dissolved in DMSO. MMQ cells were transfected with Lipofectamine 2000 (Gibco, Grand Island, NY, USA). A plasmid encoding enhanced green fluorescent protein (pEGFPN3) was from Clontech (Palo Alto, CA, USA) and the plasmid–Lipofectamine 2000 complex was added directly to cells grown in normal medium. Rat prolactin radioimmunoassay was performed as described (Ren & Braunstein 1990) with a sensitivity of 2 ng/ml.

Apoptosis assay

In experiments utilizing proteasome inhibitors, cells were plated one day before drugs were added and the cells were grown in serum-supplemented medium at all times. MMQ cells were washed twice with PBS by pelleting and resuspension, spun into 8-well Nunc chamber slides at 1300 g for 2 min and fixed with ethanol. GH3 cells were fixed with ethanol after cells were washed twice with PBS. Significant GH3 cell floating occurred after 48 h of proteasome inhibitor treatment at high concentrations, and in experiments of longer treatment durations floating cells were collected and mixed with trypsinized adherent cells and apoptosis was assayed as with MMQ cells. Nuclear morphology was determined after cells were fixed and stained with Hoechst 33342 and observed under a Nikon fluorescence microscope (Yu *et al.* 2000a). Cells with condensed, fragmented nuclei were scored as apoptotic. In each experiment, more than 400 cells were counted. The apoptotic nature of these cells was confirmed by TUNEL staining performed according to the manufacturer's recommendations (Hoffmann-La Roche, Switzerland). In our hands, TUNEL assay results were consistent with those obtained from apoptotic nuclear morphology (Yu *et al.* 2000a). Apoptosis was also assayed by DNA laddering (Drexler 1997). Cells were lysed in lysis buffer (20 mM EDTA, 100 mM Tris, 0.8% SDS, pH 8.0), and digested with RNase A (0.1 mg/ml) at 37 °C for 1 h. The lysate was further digested with proteinase K (10 mg/ml) at 50 °C for 1.5 h and extracted with phenol–chloroform twice. DNA was precipitated with ethanol, resuspended in

TE buffer and run in 1.5% agarose gel at 2 v/cm. Ethidium bromide-stained gels were photographed with a digital camera.

Cell cycle study

DNA synthesis was measured by BrdU incorporation. BrdU (40 µM) was incubated with cells at 37 °C for 1 h before cells were fixed, treated with 2 M HCl, and stained with a BrdU antibody (Dako A/S, Copenhagen, Denmark, 1:200) and rhodamine-labeled goat antimouse IgG. Cells were counterstained with Hoechst 33342. BrdU and nuclear images of the same field were separately acquired by a digital camera and analyzed with ImagePro software (Media Cybernetics, Bothell, WA, USA). The percentage of BrdU-positive cells was obtained by dividing the total number of BrdU-positive cells by the number of nuclei (defined by Hoechst staining). The mean fluorescent intensity of BrdU-positive cells was calculated by ImagePro. Cell cycle was also analyzed by flow-assisted cell sorting (FACS).

Caspase assay

Caspase was assayed as recommended by the manufacturer (Calbiochem). Cells were washed twice with PBS and lysed in a lysis buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% Triton X-100, 5 mM beta-mercaptoethanol, 1 mM PMSF, 38 µg/ml aprotinin, 8 µg/ml leupeptin) for 20 min on ice. The supernatant was collected after 10 000 r.p.m. at 4 °C for 1 min. Caspase substrate (4 mM stock in DMSO) was added to a final concentration of 160 µM, and optical density at 405 nm was continuously monitored.

Western blotting

MMQ or GH3 cells from 6-well plates were lysed in 200 µl SDS-PAGE lysis buffer and 10 µl lysate were run on 8% SDS-PAGE, transferred to PVDF membrane and probed with antibodies to ubiquitin, poly(ADP-ribose) polymerase (PARP), β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), EGFP (Clontech), or lamin B (Zymed, South San Francisco, CA, USA) at 1:1000, and appropriate peroxidase-linked second antibodies. Blots were visualized with ECL reagents (Amersham, UK).

Results

Proteasome inhibitors induce apoptosis in rat pituitary tumor cells

Incubation of cells with proteasome inhibitors (100 nM to 10 µM) resulted in nuclear condensation and fragmentation characteristic of apoptotic nuclear morphology in both

Figure 1 Rat pituitary tumor cell responses to proteasome inhibitors. (A) MMQ cells were treated with proteasome inhibitor PSI (10 μ M) for 24 h and spun onto Nunc chambers. Cells were fixed and stained with Hoechst 33342. Apoptotic cells with condensed, fragmented nuclei are evident in cells treated with PSI. Ctr, control. (B) MMQ cells treated with PSI (10 μ M) for 24 h were fixed and then stained with Hoechst and a TUNEL labeling kit. (C) MMQ cells were treated with increasing concentrations of PSI for 16 h. DNA was extracted, resolved in agarose gels, and stained with ethidium bromide. (D) MMQ cells were transfected with a plasmid encoding EGFP and treated with PSI (10 μ M) for up to 12 h. Cells were lysed and ubiquitinated proteins (Ub-proteins) were recognized by Western blotting using an antibody to ubiquitin. Equal loading was verified with an antibody to EGFP.

MMQ (Fig. 1A) and GH3 cells (data not shown). The condensed, fragmented nuclei were labeled with TUNEL signal, confirming the apoptotic nature of the cell death (Fig. 1B). MMQ cells also exhibited DNA laddering in response to proteasome inhibitor treatment (Fig. 1C). Three hallmarks of apoptosis were present in proteasome inhibitor-treated cells, indicating that the observed cell death was apoptotic in nature. Inhibition of proteasome activity was confirmed by accumulation of poly-ubiquitinated proteins after treatment with proteasome inhibitors (Fig. 1D). Three proteasome inhibitors, PSI, MG-132, and lactacystin, dose-dependently caused apoptosis in rat pituitary tumor MMQ and GH3 cells (Fig. 2). PSI was the most potent proteasome inhibitor among the three, and 10 μ M PSI caused 70% apoptosis in MMQ cells and 25% in GH3 cells in 24 h. MMQ cells

were more sensitive than GH3 to the pro-apoptotic effects of all three proteasome inhibitors. The calpain (a cysteine protease) inhibitor, ALLM, did not cause cell apoptosis, neither did 0.1% methanol nor 0.1–0.2% DMSO (data not shown)

Low concentrations of proteasome inhibitors inhibit GH3 cell growth

Since GH3 cells showed less apoptosis in response to proteasome inhibitor treatment, we studied the effects of proteasome inhibitors on GH3 cell growth. PSI at 100 nM and 1 μ M, and MG-132 at 1 μ M completely inhibited GH3 cell growth (Fig. 3). Microscopic observation of GH3 cells treated under those conditions showed extensive apoptosis after 48 h. Treatment with 10 nM PSI

Figure 2 Proteasome inhibitors dose-dependently cause apoptosis. MMQ and GH3 cells were treated with 3 proteasome inhibitors (PSI, MG-132, and lactacystin) and a calpain inhibitor, ALLM, at various concentrations for 24 h. Cells were fixed and stained with Hoechst 33342 and apoptosis was determined by fluorescence microscopy.

partially inhibited, while 10 or 100 nM MG-132 did not affect GH3 cell growth. Microscopic observation of cells did not reveal apoptosis. PRL secretion by GH3 cells followed a pattern similar to that of growth in response to

PSI or MG-132 (Fig. 3). Therefore, low doses of proteasome inhibitors inhibited pituitary tumor cell growth without causing apoptosis and did not alter PRL secretion by GH3 cells.

Figure 3 Proteasome inhibitors inhibit GH3 cell growth. GH3 cells were treated with PSI or MG-132 for 5 days. On each day before and after proteasome inhibitor treatment, total protein of adherent cells was measured and prolactin (PRL) levels in culture medium were measured by RIA. The results shown are the means $\pm$ S.E.M. of three parallel dishes in a representative experiment.

Figure 4 Proteasome inhibitors do not cause apoptosis in normal pituitary cells. GH3 cells and primary rat pituitary cells were cultured for 36 h, followed by treatment with a proteasome inhibitor, PSI or MG-132 (1 nM to 10 μ M), for 24 h. No significant cell floating was observed under these conditions. Cells were then fixed and stained with Hoechst 33342 and apoptosis was determined by microscopy. Endocrine cells in the primary pituitary culture were identified by cell size and the distinctive cell aggregate. The results shown are the means $\pm$ S.E.M. of three parallel dishes in a representative experiment.

Proteasome inhibitors spare normal pituitary cells

We tested whether proteasome inhibitors cause apoptosis in normal rat pituitary cells. GH3 cells were used as a tumor cell control because they are adherent and appear to have less apoptosis in response to proteasome inhibitors. Between two and three percent of endocrine cells in primary rat pituitary cultures exhibited apoptosis in the absence of any treatment (Fig. 4). The proteasome inhibitors PSI and MG-132, even at the highest concentrations (10 μ M), did not cause further apoptosis in these normal pituitary cells. GH3 cells, on the other hand, exhibited no apoptosis without treatment or when treated with low concentrations of proteasome inhibitors. At higher concentrations, proteasome inhibitors caused significant apoptosis in GH3 cells. The results suggest that proteasome inhibitors selectively cause apoptosis in pituitary tumor cells but spare normal pituitary cells.

Proteasome inhibitors inhibit DNA synthesis

Since key proteins involved in cell cycle regulation are degraded by the proteasome, we tested the effects of proteasome inhibitors on the cell cycle. Cell cycle components were measured with FACS. After 8 h of 10 μ M PSI treatment, there was a significant increase in cells at G2/M ($20 \pm 1\%$ versus $12 \pm 2\%$ in control cells, $P < 0.05$). At this time, apoptosis was found in $\sim 5\%$ of cells. However, after longer treatment times, there were no significant changes in the cell cycle of live cells. Since FACS measures DNA content but does not provide dynamic cell cycle information, we used BrdU incorporation to measure active DNA synthesis. After 6 h of PSI treatment, the number of BrdU-positive cells was similar to that of untreated cells, but the intensity of BrdU label

had begun to decrease, showing that the rate of DNA synthesis had already slowed (Fig. 5). After 10 h of PSI treatment, both the number and intensity of BrdU-positive cells greatly decreased, indicating that the number of cells in S phase and the DNA synthesis rate in S phase cells were both decreased. Thus PSI-treated cells lost active DNA synthesis without apparent general changes in the cell cycle.

Proteasome inhibitors activate caspases

To determine if proteasome inhibitors activate caspases, mediators of apoptosis, caspase activity was measured in lysates of proteasome inhibitor-treated cells using colorimetric caspase substrates. The lysate of PSI-treated cells showed higher caspase activity on LEHD (substrate for caspases 4, 5, 9), DEVD (substrate for caspases 3, 6, 7, 8, 10), and VDVAD (substrate for caspase 2), but not on YVAD (substrate for caspases 1, 4), WEHD (substrate for 1, 4, 5), IETD (substrate for caspase 8), or VEID (substrate for caspase 6) (Fig. 6, left panel). Therefore, specific caspases were activated by a proteasome inhibitor. The quantity of caspase substrate was also assayed in GH3 cells by Western blotting (Fig. 7). Poly(ADP-ribose) polymerase (PARP) (caspase 3 substrate) began to be degraded after 6 h of PSI treatment, as evidenced by the appearance of a PARP fragment. β -Catenin and lamin B (both substrates of caspase 6) were not changed by PSI treatment in the first 24 h, suggesting caspase 6 activation is not a primary effect of proteasome inhibitors. After 24 h, all substrates were degraded although a β -catenin fragment was intact even after 72 h. The same antibodies could not detect PARP or β -catenin from MMQ lysate but the quantity of lamin B in MMQ cells was similar to that in GH3 cells. We could not detect p53, p27, or p21 by

Figure 5 Proteasome inhibitors inhibit DNA synthesis. MMQ cells were treated with 10 μ M PSI for up to 24 h. (Top panel) At the indicated times, cells were fixed and stained with Hoechst 33342 and apoptosis was determined by microscopy. (Middle and bottom panels) 1 h before the indicated times 40 μ M BrdU were added, and at the indicated times cells were fixed and stained with an antibody to BrdU. The number of BrdU-positive cells (middle panel) and their fluorescent intensity (bottom panel) were recorded.

available antibodies in either MMQ or GH3 cells (data not shown). When cells were treated with proteasome inhibitor along with a broad-range caspase inhibitor, caspase inhibitor III, apoptosis was dramatically inhibited at every concentration of proteasome inhibitor tested (Fig. 6, right panel). These results demonstrate that specific caspases play critical roles in proteasome inhibitor-induced apoptosis.

Discussion

We show that proteasome inhibitors induce apoptosis in rat pituitary tumor cells. The fact that all three proteasome inhibitors tested caused apoptosis in two pituitary tumor cell lines suggests that the apoptotic effect on cells of pituitary tumors is a common property of the drugs and is not due to drug idiosyncrasy. For tumor therapeutics to be effective, these agents must not only ablate tumor cells, but must also spare normal cells. It is encouraging that the two proteasome inhibitors that cause most apoptosis in pituitary tumor cells did not cause apoptosis in endocrine cells of primary rat pituitary cultures, consistent with a previous report that proteasome inhibitors differentially inhibit colony formation of chronic myelogenous leukemia cells over that of normal bone marrow CD34+ cells (Soligo *et al.* 2000). When used intra-tumorally at a sufficiently high concentration, one proteasome inhibitor (PS-341) caused significant prostate cancer cell apoptosis (Adams *et al.* 1999). These results therefore suggest that proteasome inhibitors may have therapeutic potential in treating pituitary tumors. Unlike bromocriptine, the proteasome inhibitors did not affect PRL secretion.

We demonstrated that proteasome inhibitors have two therapeutically beneficial effects on pituitary tumor cells: inducing apoptosis at higher doses and inhibiting proliferation at lower doses. Nuclear morphology, TUNEL labeling, and DNA laddering strongly indicate that the observed cell death in response to proteasome inhibitors is apoptotic in nature. Caspases are mediators of apoptosis and we showed that some, but not all, caspases are activated by proteasome inhibitors. Specifically, caspases 2 (by caspase assay) and 3 (by Western blotting) are activated but caspases 6 and 8 (by caspase assay and Western blotting) are not activated by PSI. Because of poor specificity of some substrates, activation of other individual caspases was not determined. Caspase 2 is a pro-caspase which initiates caspase-mediated apoptosis, while caspase 3 is an effector caspase which cleaves gelsolin and PARP (Cryns & Yuan 1998, Thornberry & Lazebnik 1998). The importance of caspase activation is manifest by the almost complete inhibition of proteasome inhibitor-induced apoptosis by a broad-range caspase inhibitor, caspase inhibitor III. Although both initiator and effector caspases are activated by proteasome inhibitors, mechanisms of caspase activation by proteasome inhibitors are not known. Recently, an inhibitor of apoptosis was shown to promote proteasomal degradation of active caspase-3, but not pro-caspase-3, the inactive precursor of active caspase 3 (Suzuki *et al.* 2001). Proteasome inhibitors may directly upregulate the amount of active caspase-3 and initiate the caspase cascade. NF κ B is an anti-apoptotic factor and its inhibitor, I κ B, is degraded by a proteasome-mediated mechanism; thus proteasome inhibitors may induce apoptosis by inhibiting NF κ B (McDade *et al.* 1999, Cusack *et al.* 2001, Russo *et al.* 2001). The tumor suppressors, p53,

Figure 6 Proteasome inhibitors activate caspases. (Left panel) Lysates from MMQ cells were treated with PSI (100 nM) for 22 h and untreated cells were incubated with caspase substrate (160 μM) for 2 h and optical density (405 nm) of the reaction mixture was measured. Caspase activity of untreated cells was defined as 1 and the relative activity of PSI-treated cells for each substrate is shown. Gray columns signify higher caspase activity ($P < 0.001$). (Right panel) MMQ cells were incubated with PSI (100 nM to 10 μM) together with 100 μM caspase inhibitor III or 1% DMSO for 24 h. Cells were fixed and stained with Hoechst 33342 and apoptosis was determined by fluorescence microscopy.

p27, and p21, are degraded by the proteasome, and proteasome inhibitors upregulate their intracellular concentration (Lopes *et al.* 1997). Apoptosis caused by proteasome inhibitors may be secondary to accumulation of these tumor suppressors. An early study suggests that p53 may mediate proteasome inhibitor-induced apoptosis (Lopes *et al.* 1997). Since many proteins are degraded by proteasome and these should all be upregulated by proteasome inhibitors, it is unlikely that accumulation of a single protein would cause apoptosis. Later studies actually show that p53 itself is not critical for proteasome inhibitor-induced apoptosis in other systems (Herrmann *et al.* 1998, Soligo *et al.* 2000). We could not detect p53, p27, or p21 in GH3 cells, but apoptosis nonetheless still occurred. The same reasoning can be applied to inhibition of pituitary tumor cell proliferation by proteasome inhibitors. It may be that the blockade of protein degradation constitutes a general stress that reduces cell proliferation.

The two cell cycle effects of proteasome inhibition, G2/M blockade and DNA synthesis inhibition, both suggest cell cycle arrest. Similar G2/M blockade is caused by overexpression of pituitary tumor transforming gene (PTTG), a protein involved in regulating sister chromatid separation and which is degraded by the proteasome (Zou *et al.* 1999, Yu *et al.* 2000b). It will be interesting to study whether G2/M blockade caused by proteasome inhibitors depends on PTTG. Although proteasome inhibitors inhibited DNA synthesis, apoptosis does not appear to depend on DNA synthesis inhibition, because no G1/S block was found in live cells after proteasome inhibitor treatment. Actually, the cell cycle components of live cells

Figure 7 Proteasome inhibitors cause specific changes in caspase substrates. GH3 cells were treated with PSI (10 μM) for up to 72 h. At the indicated interval, floating cells were collected and mixed with trypsinized adherent cells and apoptosis was determined by fluorescence microscopy as for MMQ cells (Materials and Methods). The quantity of the caspase substrates poly(ADP-ribose) polymerase (PARP), β-catenin, and lamin B was determined by Western blotting. Ub-proteins, ubiquitinated proteins.

after prolonged treatment were similar to those of control cells, further implying that cells die at every phase of the cell cycle.

Leukemia, prostate, pancreatic, and lung cancer cells are all susceptible to the apoptotic effect of proteasome inhibitors (Adams *et al.* 1999, McDade *et al.* 1999, Soligo *et al.* 2000, Sun *et al.* 2001), and this effect is not exclusive to pituitary tumor cells. Furthermore, proteasome inhibition of nontumorous proliferating endothelial cells also results in apoptosis (Drexler *et al.* 2000). Proliferating fibroblasts in primary rat pituitary cell cultures were indeed susceptible to proteasome inhibitors (data not shown), but endocrine pituicytes are resistant. Therefore, proteasome inhibitors seem to preferentially cause apoptosis in proliferating cells.

In summary, we have shown that proteasome inhibitors cause apoptosis in rat pituitary tumor cells while normal pituitary endocrine cells are spared. The results suggest that proteasome inhibitors are potentially novel agents for treating pituitary tumors not amenable to other treatments.

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